

Bitting Deterrent Activity of a Deet Analog, Two DEPA Analogs, and SS220 Applied Topically to Human Volunteers Compared with Deet Against Three Species of Blood-Feeding Flies

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ABSTRACT An earlier in vitro screening of *N,N*-diethyl-3-methylbenzamide (Deet) and *N,N*-diethylphenylacetamide (DEPA) analogs showed that two DEPA analogs, *N,N*-diethyl(3-bromophenyl)acetamide and *N,N*-diethyl[(α,α,α-trifluoro-*m*-tolyl)]acetamide, and one Deet analog, *N,N*-diethyl[3-(trifluoromethyl)]benzamide, had biting-deterrent activities that were superior to Deet against *Aedes aegypti* (L.) and *Anopheles stephensi* Liston. In the current study, the three analogs and (1*S*,2'*S*)-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220) were applied topically to the skin of human volunteers at 24 nmol compound/cm² skin and compared with the activity of Deet at the same dose against biting by *Ae. aegypti*, *An. stephensi*, and *Phlebotomus papatasi* Scopoli females. SS220 proved to be as effective as Deet against *Ae. aegypti* and *P. papatasi* but more effective than Deet against *An. stephensi*. Contrary to the earlier in vitro tests, results with humans (in vivo testing) showed that neither of the DEPA analogs nor the Deet analog performed more effectively than Deet against the insects. The in vivo results showed that the analogs were not sufficiently effective to warrant further development. Notably, in vivo and in vitro methods used in discovery of personal protection chemicals for human use against blood-feeding flies can both be effective discriminating tools, but results obtained with the respective methods may not always agree. Ultimately, we surmise that in vivo testing methods with humans must be used to discriminate among compounds that superficially seem effective when screened using an in vitro method.

KEY WORDS *N,N*-diethyl-3-methylbenzamide, (1*S*,2'*S*)-methylpiperidinyl-3-cyclohexene-1-carboxamide, *N,N*-diethyl(3-bromophenyl)acetamide, *N,N*-diethyl[(α,α,α-trifluoro-*m*-tolyl)]acetamide, *N,N*-diethyl[3-(trifluoromethyl)]benzamide

Ma et al. (1999) reported that the electronic properties of the amide group in analogs of the well-known repellent *N,N*-diethyl-3-methylbenzamide (Deet) seemed to be key to the duration of a molecule's activity against mosquito bites. Their calculations indicated that the numerical values of the amide

electronic properties fell into discrete ranges associated with long-duration mosquito protection for a compound. These optimal values were used to guide the synthesis of eight Deet analogs and nine DEPA analogs as new potential insect repellents. Debboun and Wagman (2004) used an in vitro blood-feeding system developed by Rutledge et al. (1976) to evaluate the electronically designed candidate repellents. The biological screening involved replicated dose-response assays against *Aedes aegypti* (L.) and *Anopheles stephensi* Liston conducted over a period of several years. The in vitro assay results indicated that *N,N*-diethyl[(α,α,α-trifluoro-*m*-tolyl)] acetamide (DM156), *N,N*-diethyl(3-bromophenyl) acetamide (DM34), and *N,N*-diethyl[(trifluoromethyl)] benzamide (DM159) seemed to be more effective than Deet against the two species of mosquitoes. These compounds were selected for further evaluation by using human volunteers to determine whether they should be developed for practical use as personal protective measures against mosquitoes and sand flies.

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Materials and Methods

Chemicals. Deet was obtained from Morflex, Inc. (Greensboro, NC) and (1S,2'S)-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220) was synthesized earlier at the Chemical Affecting Insect Behavior Laboratory, U.S. Department of Agriculture, Beltsville Agricultural Research Center, Beltsville, MD, and it was of 95% stereoisomeric purity and >99% chemical purity (Klun et al. 2003). DM159, DM34, and DM156 were synthesized as described below under Analytical Methods. The reagents for these syntheses were purchased from Aldrich (Milwaukee, WI).

Analytical Methods. Gas chromatography analyses for chemical purity determination were carried out in a split mode on a Shimadzu GC-17A with flame ionization detector fitted with a DB-5 column, 15 m \times 0.25 mm, film thickness 0.25 μm (J&W Scientific, Folsom, CA). Hydrogen was used as carrier gas at 35 cm/s linear velocity. Electron-ionization mass spectra (70 eV) were obtained with a 5973 mass selective detector (Agilent Technologies, Palo Alto, CA) interfaced with 6890 N gas chromatograph system equipped with a 30 m \times 0.25 mm i.d. \times 0.25- μm film HP-5MS column. Helium was used as the carrier gas at 1 ml/min. All three test amides were synthesized using a standard acyl chloride-amine methodology described in detail by Klun et al. 2003.

N,N-Diethyl[3-(trifluoromethyl)]benzamide (DM159) was prepared from (trifluoromethyl)benzoyl chloride and diethylamine in 93% yield. The amide was purified by distillation at 112–115°C/0.45 mmHg and was 99.7% pure. Mass spectrometry (MS) (m/z , %): 245 (18%, M^+), 244 (33), 173 (100), 145 (32).

N,N-Diethyl(3-bromophenyl)acetamide (DM34) was synthesized in 70% yield and 97.6% purity by converting 3-bromophenylacetic acid to the corresponding acyl chloride and reacting the latter with diethylamine. Boiling point (b.p.) 162–165°C/0.3 mmHg. MS (m/z , %): 271 and 269 (both 24%, M^+), 171 (14), 169 (14), 100 (100), 90 (12), 89 (13).

N,N-Diethyl[(α,α,α -trifluoro-*m*-tolyl)]acetamide (DM156) was prepared in 90% yield and 99.7% purity from (α,α,α -trifluoro-*m*-tolyl)acetic acid as described for DM 34. b.p. 124°C/0.35 mmHg. MS (m/z , %): 259 (27%, M^+), 240 (4, $\text{M}^+ - \text{F}$), 159 (44), 100 (100), 71 (66), 58 (23), 44 (24).

Insects. *Ae. aegypti* (red eye Liverpool strain), *An. stephensi* [Delhi strain, traced by Shute and Maryon (1960) to a colony established in 1947], and *Phlebotomus papatasi* Scopoli (Israeli strain, obtained from Hebrew University, Jerusalem, Israel, in 1983) used in the study were from human-pathogen-free colonies maintained at the Department of Entomology, Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. The mosquitoes were reared using the procedure of Gerberg et al. (1994). The larvae were fed a diet of ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA; www.tetra-fish.com). Adult insects were maintained in a photoperiod of 12:12 h (L:D) h with lights on at 0600 hours) at 27°C and 80% RH with cotton pad moistened

with 10% aqueous sucrose solution. Mated nulliparous *Ae. aegypti* and *An. stephensi* females (5–15 d old) were tested. *An. stephensi* had access to water 24 h before testing and *Ae. aegypti* had no water 24 h before testing. *P. papatasi* was reared using methods described by Modi and Rowton (1999). The nulliparous females were 1–3 d old before being used in the bioassays.

Bioassays. In vivo bioassays involved two 25–30-yr-old females and five 26–62-yr-old male Caucasian volunteers treated with the three DM compounds, Deet, and SS220, and exposure to feeding mosquitoes and sand flies. We followed the guidelines established by the National Institutes of Health for tests involving humans, and protocols were approved by the Human-Use Review Board (Human Use Protocol No. 0852, HSSRB No. A-10337) of the WRAIR. The Deet analog and the DEPA analogs were studied toxicologically and approved for entomological testing using humans (Snodgrass et al. 2001a,b,c), and SS220 and Deet had abundant biological safety clearances (Klun et al. 2003). Bioassays were conducted with the volunteers by using Klun & Debboun (K & D) modules and methods described by Klun and Debboun (2000).

Volunteers wearing short pants were seated. Using a skin-marking template and a washable-ink marker, skin areas representing the 3- by 4-cm floor openings of six cells of the K & D module were outlined on the outer, top, and inner thigh positions of each leg. The six treated cell rectangles each represented a randomized block, and each volunteer had three blocks on each of two thighs. All treatments against the mosquitoes *Ae. aegypti*, *An. stephensi*, and the sand fly *P. papatasi* were pipetted onto a 4- by 5-cm rectangular area (so the area of skin covered by a treatment exceeded the template marks by 0.5 cm in every direction) of the subjects' skin with 55 μl of ethanol/treatment containing 8.73 nmol compound/ μl ethanol. Treating a slightly larger area ensured that the areas beneath each K & D module cell contained only treated skin. The applications yielded stoichiometrically equivalent doses of 24 nmol/cm² skin for each compound. This dose was used because previous work with Deet and SS220 showed that it would be sufficient to suppress biting by at least 80% relative to untreated skin (Klun et al. 2003). Skin treated with ethanol alone served as control.

In all tests, adjacent cells of the K & D modules were provided each with five female mosquitoes or sand flies randomly selected from cages containing \approx 200 adults. The insect-charged K & D module was positioned over the treated skin areas, trap doors above the areas opened, and the number of females biting (proboscis inserted into skin and/or observed blood engorged) within each of the cells was recorded in a 2-min skin exposure, and then trap doors were closed. Females were recorded as either having bit or not during a trial. The K & D in vivo bioassay measured the biting (feeding) deterrent behavioral effects (Dethier et al. 1960, Klun et al. 2006) of the two DEPA analogs (DM34 and DM156), Deet analog (DM159), SS220, and Deet applied topically to skin. In this laboratory

Table 1. Proportion of mosquitoes and sand flies not biting on human volunteer skin individually treated with five compounds

Insect	Control	Proportion insects not biting (SE)					n
		Deet	SS220	DM159	DM156	DM34	
<i>P. papatasi</i>	0.23 (0.03)	0.91 (0.02)a	0.93 (0.02)ab	0.74 (0.04)a	0.93 (0.02)ab	0.65 (0.04)a	150
<i>An. stephensi</i>	0.18 (0.05)	0.65 (0.06)a	0.80 (0.05)a	0.72 (0.06)ab	0.68 (0.06)ab	0.55 (0.06)a	60
<i>Ae. aegypti</i>	0.15 (0.03)	0.82 (0.03)a	0.89 (0.03)ab	0.68 (0.04)a	0.59 (0.04)a	0.38 (0.04)a	150

n is number of insects tested against each compound at a dose of 24 nmol compound/cm² skin; SE is standard error. Proportions followed by a, significantly different from control; proportions followed by b, not significantly different from Deet ($\alpha = 0.01$).

study, we consider the terms biting and feeding to be synonymous.

Tests with *Ae. aegypti* involved one female and two male volunteers, and 150 mosquitoes were used to test each treatment. Tests with *P. papatasi* involved one female and three males, and 150 sand flies were used to test each treatment. Testing with *An. stephensi* used two male volunteers, and 60 mosquitoes were used to test each treatment. The bioassay tests were done in a walk-in incubator (27°C and 80% RH) in ambient fluorescent light from 0730 to 1030 hours. Insects were used once in a test and then discarded.

Tests with the three species of insects were conducted in different time periods. A logistic regression approach (Klun et al. 2003) was used to model the proportion of nonbiting mosquitoes jointly for each group of compounds (including the control). We used the NLMixed procedure in SAS Institute (1999) to produce estimates, their standard errors, and tests of significance. As in previous studies (Klun et al. 2003), we found no indication that a block or leg effect was important, so these effects were ignored in the analysis. However, as in previous studies, there were volunteer-to-volunteer differences. We modeled these volunteer effects as draws from a normal distribution, making this a generalized mixed (random and fixed effects) model with a binomial link (McCulloch and Searle 2001). Rather than make all possible comparisons of compound pairs (with a subsequent loss of power), two sets of 1 df contrasts (*t*-tests) were made with the control and with Deet. The level of significance was set at $\alpha = 0.01$ rather than the usual $\alpha = 0.05$ to avoid problems from making multiple comparisons (each compound was tested twice and this induces a statistical correlation that affects the true α values).

Results and Discussion

The bioassay results presented in Table 1 show that the feeding deterrent performance of most compounds varied from one species to another. Against *P. papatasi*, SS220, Deet, and DM156 were statistically indistinguishable. However, DM159 and DM34 were significantly more effective than untreated control skin, but significantly less effective than SS220, Deet, and DM156. Against *An. stephensi*, SS220 was significantly more effective than all other compounds; Deet, DM159, DM156, and DM34 were statistically indistinguishable but significantly more effective than untreated skin. Against *Ae. aegypti*, SS220 and Deet were equally effective in deterring feeding and significantly

more effective than any DEPA or Deet analog. DM159 was more effective than both DM156 and DM34, but these two analogs were more effective than untreated skin. Overall, this study with humans showed that the Deet analog and the DEPA analogs were not more effective than Deet in deterring biting of the three blood-feeding flies and demonstrated that they were not efficacious enough to warrant further evaluation and development.

Although it is an arguable assumption, and our bioassay data do not absolutely prove it, we surmise that human testing of candidate repellent and feeding deterrent compounds must be a final step in identifying new chemical tools for protection against arthropods that vector human diseases (Klun et al. 2005). It is encouraging to note that, although the DM analogs did not perform better than Deet as predicted by Deb-boun and Wagman (2004), the compounds generally possessed activity that was significantly superior to untreated skin. This outcome is evidence that the Rutledge et al. (1976) in vitro blood-feeding test system has merit as a screening tool. The general problem is that in vitro and in vivo tests results do not always closely agree (Rutledge and Gupta 2004, Klun et al. 2005). This complicates the discovery and development of new chemicals to protect humans from arthropods that vector diseases. For the future, a continued effort to develop test methods that bring in vitro and in vivo assay results into closer agreement is a daunting but worthy endeavor.

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